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Identification of *Mycobacterium* species following growth detection with the BACTEC MGIT 960 system by DNA line probe assay

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ABSTRACT

Background: The tuberculosis and infections caused by nontuberculous mycobacterial (NTM) species are increasing in patients presented with respiratory illness, and it is crucial to document the epidemiology of these infections.

Objectives: To study the mycobacterial species and *in vitro* drug susceptibility trends of *Mycobacterium tuberculosis* found in the respiratory specimens.

Materials and methods: A prospective descriptive study from July 2009 to December 2012. The BACTEC MGIT system tubes with growth were used in the study. GenoType *Mycobacterium* (Hain Diagnostika, Nehren, Germany) assays were used to identify the mycobacteria. The drug susceptibility testing was performed by the MGIT 960 system.

Results: A total of 1745 MGIT 960 system positive tubes were included. *M. tuberculosis* complex (MTC) constituted 67.45% of the yield isolated, 30.83% were nontuberculous mycobacterial species, 0.17% were *Mycobacterium bovis* BCG and 1.55% were not interpretable to species levels. *Mycobacterium fortuitum* (45.71%), *Mycobacterium abscessus* (26.21%) and *Mycobacterium intracellulare* (10.41%) were major NTM identified. The drug susceptibility study showed that 6.88% (81/1177) of MTC were drug-resistant TB, 56 isolates were resistant to one of the first-line anti-TB drugs, 25 isolates were found to be resistant to 2 or more first-line anti-TB drugs, of which 19 (20.46%) were MDR-TB and one of the isolates in the year 2011 was confirmed XDR-TB.

Conclusion: *M. tuberculosis*, *M. fortuitum*, *M. abscessus* and *M. intracellulare* were major mycobacterial species detected in the respiratory samples. The drug susceptibility testing showed that the majority of MTC were sensitive to first-line anti-TB drugs.

Introduction

Mycobacterium is a genus of *Actinobacteria* in the family *Mycobacteriaceae*. For diagnostic and treatment purposes, the mycobacteria can be broadly divided into: *Mycobacterium tuberculosis* complex (MTC), which can cause tuberculosis (TB); *Mycobacterium leprae* which causes leprosy, and nontuberculous *mycobacteria* (NTM).

M. tuberculosis is a species of *M. tuberculosis* complex that causes TB. TB is one of the world's deadliest diseases with over 9 million people becoming sick, and about 2 million TB-related deaths worldwide have been reported [1]. In 2009,

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there were an estimated 13.7 million chronic active cases, 9.3 million new cases, and 1.8 million deaths, mostly in developing countries [2]. However, the TB incidence rates are falling globally; by 2012, the TB mortality rate had been reduced by 45% since 1990 [3].

TB is a communicable disease; it spreads by respiratory droplets from a person with an active disease which are then inhaled by another person. The diagnosis can only be made by demonstrating the presence of tubercle bacilli in the sputum by means of microscopy and/or culture in the laboratory. Direct microscopy is a simple, inexpensive, and reliable technique for the detection of cases of pulmonary tuberculosis. However, it has poor sensitivity in extra-pulmonary tuberculosis, and it cannot distinguish viable from nonviable organisms and different mycobacterial species. It has been reported that between 5000 and 10,000 tubercle bacilli per milliliter of sputum is required to give a positive result.

Conventional culture by solid Löwenstein–Jensen (LJ) medium for mycobacterial growth is slow, requiring a 3–8 week period of incubation. Most of the laboratories are using a fully automated mycobacterial liquid culture BD BACTEC MGIT 960 System for rapid detection of mycobacterial growth and to increase overall mycobacterial recovery from clinical specimens. Culture still remains the gold standard, and every attempt should be made to isolate the organism from specimens received.

Traditionally, the mycobacterial isolates are identified to the species level by a series of phenotypic and biochemical tests. These tests are slow and the interpretation of results may vary; the molecular biology techniques based on the PCR and reverse hybridization procedure are therefore the preferred methods in the mycobacterial identification. Several commercial systems using various technologies for the detection and identification of *Mycobacterium* spp. are in routine use, including DNA hybridization-based AccuProbe *M. tuberculosis* complex (MTBC) assay (TB AccuProbe; Gen-Probe, Inc., San Diego, Calif.) based on species-specific DNA probes, the INNO-LiPA MYCOBACTERIA line probe assay (Innogenetics, Ghent, Belgium) based on the nucleotide differences in the 16S-23S rRNA spacer region, GenoType *Mycobacterium* (Hain Diagnostika, Nehren, Germany) based on DNA hybridization technology on nitrocellulose strips, the Cobas Amplicor PCR system (Roche Molecular Diagnostics, Basel, Switzerland), and the GEN-PROBE AMPLIFIED *M. tuberculosis* Direct (MTD) Test (Gen-Probe Inc., San Diego, Calif.) based on transcription-mediated amplification and the hybridization protection assay to qualitatively detect *M. tuberculosis* ribosomal ribonucleic acid. Most of these systems can be used to identify mycobacteria directly from positive BACTEC, MGIT liquid cultures bottles or from the cultures [4,5].

The current TB diagnostic algorithm in the Mycobacteriology Laboratory, University Malaya Medical Centre included fluorescence microscopy using auramine–rhodamine stain, culture by both BACTEC MGIT liquid culture and solid Löwenstein–Jensen medium. The growth in the MGIT broth was identified by molecular line probe assays. The BACTEC MGIT 960 system for *in vitro* drug susceptibility testing for mycobacteria (SIRE) was used to determine the susceptibility patterns of four commonly used first-line anti-tuberculous drugs – streptomycin, isoniazid, rifampicin and ethambutol – and seven second-line anti-tuberculous drugs – amikacin, capreomycin, kanamycin, ofloxacin, ciprofloxacin, ethionamide and para-amino salicylic acid.

The objective of this study was to identify the mycobacterial species found in the respiratory specimens for a better understanding of the epidemiology of the mycobacterial diseases. Only the first MGIT liquid culture tube of each patient was included in the analysis; the subsequent repeated examinations were excluded in this study. The mycobacterial species found to be positive in BACTEC MGIT liquid culture were identified using GenoType *Mycobacterium* assays (Hain Diagnostika, Nehren, Germany) and *in vitro* drug susceptibility testing were carried out using the BACTEC MGIT 960 system.

Materials and methods

A prospective descriptive study took place from July 2009 to December 2012, and 1745 MGIT system tubes with growth detected by the instrument were included in the study. On the day of detection of growth in the MGIT 960 instrument, a 0.1 mL aliquot of the MGIT broth was removed and examined with a Ziehl-Neelsen stain to confirm the presence of acid-fast bacteria (AFB) and a gram-stain to ascertain there was no contamination with bacteria. After the confirmation of AFB, 1 ml aliquot of the MGIT broth was transferred to a screw-capped 1 mL tube for GenoType *Mycobacterium* assays (Hain Diagnostika, Nehren, Germany). The tube was first centrifuged at 15,000*g* for 15 min. The supernatant was discarded; the pellet was re-suspended with 100 μl of ultra-pure distilled water. The tube was vortexed and placed in a heating block for 30 min. The tube was then centrifuged at 15,000*g* for 5 min; 5 μl of the supernatant was re-suspended in 35 μl of PNM reagent. The DNA extraction, amplification, and hybridization of the PCR products to the strips, detection and interpretation of the results were performed according to the manufacturer's instructions. All the procedures were carried out in the BSL 3 laboratory.

The GenoType CM was first used to identify the mycobacterial species (*Mycobacterium avium* subspecies, *Mycobacterium chelonae*, *Mycobacterium abscessus*, *Mycobacterium fortuitum*, *Mycobacterium gordonae*, *Mycobacterium intracellulare*, *Mycobacterium scrofulaceum*, *Mycobacterium interjectum*, *Mycobacterium kansasii*, *Mycobacterium malmoense*, *Mycobacterium marinum–Mycobacterium ulcerans*, *Mycobacterium peregrinum*, the *M. tuberculosis complex*, and *Mycobacterium xenopi*) isolated in the Mycobacteriology laboratory. If the assay failed to identify the AFB of the MGIT system tubes, the GenoType AS was used to probe a series of additional NTM (*Mycobacterium simiae*, *Mycobacterium mucogenicum*, *Mycobacterium goodie*, *Mycobacterium cellatum*, *Mycobacterium smegmatis*, *Mycobacterium genavense*, *Mycobacterium lentiflavum*, *Mycobacterium heckeshornense*, *Mycobacterium szulgai*, *Mycobacterium phlei*, *Mycobacterium hemophilum*, *M. kansasii*, *M. ulcerans*, *Mycobacterium gastri*, *Mycobacterium asiaticum*, and *Mycobacterium shimoidei*) not covered in the GenoType CM. GenoType MTBC was used to further characterize the M. tuberculosis complex (*Mycobacterium africanum*, BCG, *Mycobacterium bovis* subspecies bovis, *M. bovis* subspecies caprae, *Mycobacterium microti* and *M. tuberculosis*) identified among children below 5 years of age.

The drug susceptibility testing was performed using the MGIT 960 system. All *M. tuberculosis* was tested for susceptibility to the four first-line drugs with critical concentration of streptomycin (Stret) 1.0 microg/ml, isoniazid (Inh) 0.1 microg/ml, rifampicin (Rif) 1.0 microg/ml, and ethambutol (Etham) 5.0 microg/ml as recommended by the manufacturer. The isolates resistant to single first-line anti-TB drugs or both isoniazid and rifampicin were tested for seven second-line drugs; the critical concentrations were amikacin 1.0 microg/ml, capreomycin 2.5 microg/ml, kanamycin 2.5 microg/ml, ofloxacin 2.0 microg/ml, ciprofloxacin 1.0 microg/ml, ethionamide 5.0 microg/ml and para-amino salicylic acid 4.0 microg/ml.

Results

A total of 1,745 MGIT 960 system positive tubes were included in this study. *M. tuberculosis complex* was the major mycobacterial species identified in the positive growth MGIT tubes. It constituted 67.45% (1177) of all the mycobacteria isolated in this study. Nontuberculous mycobacterial species made up 30.83% (538), 3 (0.17%) of the isolates were identified as *M. bovis* BCG. Twenty-seven isolates (1.55%) were not interpretable and were reported as mycobacterial species.

Seventeen species of nontuberculous mycobacteria were identified among the 538 isolates. *M. fortuitum* (45.71%), *M. abscessus* (26.21%), *M. intracellulare* (10.41%), *M.* mucogenicum (4.28%), *M. avium* (2.60%) and *Mycobacterium kansaii* (2.42%) were the major species identified (Table 1).

The drug susceptibility study of 1177 MTC indicated that drug resistant TB was not high among the patients with tuberculosis. The majority (93.12%) of the MTC were susceptible to all four first-line drugs tested. Only 81 (6.88%) MTC were found to be drug-resistant TB. The drug-resistant TB detected from 2009 to 2012 was 10.72%, 5.20%, 7.28% and 5.86%, respectively (Table 2). Among the drug-resistant TB, 69.14% (56 isolates) were resistant to one of the first-line anti-TB drugs: isoniazid, rifampin, ethambutol, or streptomycin and 30.86% (25 isolates) were found to be resistant to two or more first-line anti-TB drugs (Table 2). Mono-resistant to Isoniazid was detected in 44.64% of mono-drug resistant TB; this was followed by ethambutol (33.93%), rifampin (10.72%) and streptomycin (10.72%). Among the MTC resistant to two or more first-line anti-TB drugs, 6 (24.00%) were not MDR-TB (three were resistant to streptomycin & ethambutol and three were resistant to streptomycin and isoniazid), 19 (76.00%) were MDR-TB and 1 of the isolates in the year 2011 was a confirmed case of XDR-TB. Among the 19 MDR-TB, 8 were resistant to isoniazid and rifampicin; 6 were resistant to isoniazid, rifampicin and streptomycin; 4 were resistant to isoniazid, rifampicin and ethambutol, and 1 was resistant to all the first-line drugs (Table 2). Thirteen (68.42%) of the MDR-TB cases were sensitive to all the second-line anti-TB drugs tested, 2 (10.53%) were resistant to single second-line anti-TB drugs, and 3 (15.79%) were resistant to 2 or more second-line anti-TB drugs. The single MDR-TB isolate that showed resistant to all the second-line anti-TB drugs was confirmed to be the first XDR-TB detected by the laboratory (Table 3).

Discussions

Tuberculosis remains a leading cause of morbidity and mortality in developing countries. Similarly, from the global perspective, the infections caused by NTM are increasing [6] even though the epidemiology of nontuberculous mycobacterial infection in patients presented with respiratory illness remains poorly documented [7]. NTM are widely distributed

Table 1 – Mycobacterial species isolated from respiratory specimens: 2009–2012.

Mycobacterial species	2009	2010	2011	2012	Total (%)
M. tuberculosis complex	168	192	357	460	1177(67.45)
Nontuberculous mycobacteria					538(30.83)
M. fortuitum	19	29	102	96	246(45.71)
M. abscessus	15	15	53	58	141(26.21)
M. intracellulare	7	13	16	20	56(10.41)
M. mucogenicum	0	0	7	16	23(4.28)
M. avium	5	1	2	6	14(2.60)
M. kansaii	1	0	6	6	13(2.42)
M. chelonae	2	3	2	2	9(1.67)
M. gordonae	2	0	0	5	7(1.30)
M. scrofulaceum	0	2	2	3	7(1.30)
M. interjectum	0	0	1	5	6(1.12)
M. smegmatis	0	0	3	2	5(0.93)
M. szulgai	0	0	0	3	3(0.56)
M. goodii	0	0	1	1	2(0.37)
M. genavense	0	0	0	2	2(0.37)
M. celatum	0	0	0	2	2(0.37)
M. lentiflavum	0	0	0	1	1(0.19)
M. peregrinum	0	0	0	1	1(0.19)
M. bovis BCG	0	0	3	0	3(0.17)
Mycobacterial species	6	0	11	10	27(1.55)
Total					1745

Table 2 – Anti-TB drug susceptibility testing of MTC.

Anti-TB drug resistant patterns	2009	2010	2011	2012	Total (%)
Normal *M. tuberculosis*	150	182	331	433	1096(93.12)
Drug resistant *M. tuberculosis*	18	10	26	27	81(6.88)
Monodrug-resistant					56(69.14)
Strept	2	3	0	1	6(10.72)
Inh	4	1	5	15	25(44.64)
Rif	1	0	1	4	6(10.72)
Etham	3	0	10	6	19(33.93)
Polydrug-resistant					25(30.86)
Strept + Etham	3	0	0	0	3(12.00)
Strept + Inh	1	2	0	0	3(12.00)
Inh + Rif	4	1	3	0	8(32.00)
Inh + Rif + Strept	0	3	3	0	6(24.00)
Inh + Rif + Etham	0	0	3	1	4(16.00)
Inh + Rif + Etham + Strept	0	0	1	0	1(4.00)

Table 3 – Second-line anti-TB drug susceptibility testing of MDR-TB.

Second-line anti-TB drugs							Year				Total
ETN	AN	CAP	KANA	OFLO	CIP	PAS	2009	2010	2011	2012	
S	S	S	S	S	S	S	4	2	6	1	13
R	S	S	S	S	S	S			1		1
S	S	R	S	S	S	S		1			1
R	S	S	S	S	R	S			1		1
S	S	S	S	S	R	R			1		1
R	R	S	R	S	S	S		1			1
R	R	R	R	R	R	R			1		1
Total							4	4	10	1	19

ETN, ethionamide; AN, amikacin; CAP, capreomycin; KANA, kanamycin; OFLO, ofloxacin; CIP, ciprofloxacin; PAS, para-amino salicylic acid.

in the environment, especially in soil and water; the isolation rates of NTM from the soil, natural and treated water are remarkably similar in diverse geographic areas, but *M. kansasii*, *M. xenopi*, and *M. simiae* are recovered almost exclusively from municipal water sources and rarely from other environmental sources [8,9]. Because of NTM omnipresence in the environment and there is no evidence of human-to-human transmission [10], the isolation of NTM from the respiratory samples does not, per se, indicate infection or clinical significance. As this study is impeded by the lack of clinical information from patients, theprimary focus was on the studying of the epidemiology and diversity of the mycobacterial species inclusive of anti-TB drug susceptibility patterns of *M. tuberculosis* isolated from the respiratory specimens.

NTM diseases have been reported in most industrialized countries [11]. Currently, much of the epidemiological data of pulmonary NTM disease come from industrialized countries, mainly Europe, North America, Australia and Japan [12]. The lack of epidemiological data from the developing countries could be due to the lack of laboratory methods capable of identifying nontuberculous mycobacterial species. The availability of commercial reverse hybridization assay (GenoType Mycobacteria assays, Hain Lifescience) offers an unprecedented opportunity to many laboratories to identify at least 30 different nontuberculous mycobacterial species, and this will make possible for many laboratories to identify the mycobacterial isolates to species levels. *M. avium, M. intracellulare, M. malmoense* and *M. kansasii* have been recognized as important opportunistic pathogens in patients with human immunodeficiency virus infection; it is important to differentiate between *M. tuberculosis* complex species and NTM, as this would allow clinicians to make decisions on optimum drug therapy and implementation of appropriate infection control measures in the hospitals.

M. avium complex (MAC) was the most common NTM species causing pulmonary disease in most series of reports; in other NTMs, such as *M. abscessus*, *M. scrofulaceum, M. kansasii* and *M. xenopi*, the frequency of isolation appeared to be similar in most industrial countries [11]. Won-Jung et al. [13] reported that *M. avium* complex ($n = 94$, 48%) and *M. abscessus* ($n = 64$, 33%) were 2 nontuberculous mycobacterial species isolated from respiratory specimens in South Korea. Simons et al. [12] reported that *M. avium* complex was the most frequently isolated NTM species based on a literature search of articles published about NTM species isolated from pulmonary samples from persons in 20 countries in Asia during the period March 2009–December 2009. The second most frequently isolated species in the region was rapidly growing mycobacteria (*M. fortuitum* complex, *M. abscessus, M. chelonae*); other NTM species also frequently isolated were *M. kansasii* and *M. gordonae*. They also reported that rapidly growing mycobacteria were identified more frequently in pulmonary samples from Taiwan, China and Singapore, while *M. malmoense* and *M. xenopi* were reported only in India. Haverkort [14]

reported that the rate of NTM infections in Australia was 1.8 cases per 100,000 population, the most common group of mycobacteria isolated from respiratory specimens was *M. avium* complex, and other NTMs frequently isolated include the rapidly growing mycobacteria *M. fortuitum–M. abscessus–M. chelonae* group. In a similar report, Haverkort also highlighted the geographical differences, notably the predominance of *M. haemophilum* from Western Australia, and *M. ulcerans* from Victoria and Queensland.

The present study demonstrated the high frequency of isolating rapidly growing mycobacteria from the respiratory specimens which is inconsistent with global reports [12,14]. The most common rapidly growing mycobacteria isolated in this study was *M. fortuitum* (244/537, 45.4%), followed by *M. abscessus* (142/537, 26.4%). Most studies indicated that MAC is the most frequently isolated species in the respiratory samples. MAC is an acid-fast atypical mycobacterium that belongs to group III in the Runyon classification of nontuberculous mycobacteria. It is primarily a pulmonary pathogen that affects individuals who are immunocompromised (e.g., from AIDS, hairy cell leukemia, immunosuppressive chemotherapy) and occurs rarely in immunocompetent hosts [15]. In this study, *M avium* made up only 2.61% and *M. intracellulare* (10.4%); the figures are significantly lower as compared with reports by many investigators. *M. malmoense* and *M. xenopi*-mycobacterial species regularly encountered in Canada, England and northwestern Europe [16] but uncommon in Asia–were not identified among the NTM isolated in this study.

Among all the NTM identifiable by GenoType assays, all have been reported as human pathogens. Although the GenoType assay was able to identify about 30 different species of NTM, there were 27 isolates not interpretable suggesting that additional molecular assays are needed to study the diversity of mycobacterial species present in the environment. The detection of *M. bovis* BCG in the respiratory samples indicated the importance of differentiating this isolate to NTM and MTC in the laboratory, especially in young children with pulmonary or disseminated mycobacterial infections.

The emergence and spread of multi-drug resistant tuberculosis (MDR-TB) is a worldwide problem threatening to destabilize global tuberculosis control. The prevalence of MDRTB is increasing throughout the world; the proportion of multidrug resistance among the newly diagnosed TB and previously treated cases reported between 2007 and 2010 ranged from 0% to 28.9% and 0% to 65.1%, and the proportion of MDR-TB cases with extensively drug resistance (XDR-TB) between 2007 and 2010 was 9.4% [17]. WHO Tuberculosis Control in the SEA Region 2012 [18] reported that among the SEA Region member countries, the MDR-TB rate is estimated to be at around 17% (range: 17–18%) among previously treated cases; among newly detected cases, MDR-TB incidence rates are lower (1.7–2.5%). These incidence rates translate to 105,000 MDR-TB cases (85,000–125,000), accounting for nearly one fourth of the world's MDR-TB cases that were estimated to exist among notified cases in 2010. The estimated MDR-TB rates were highest in Thailand (34.5% among previously treated cases) and Bangladesh (28% among previously treated cases). Bhutan, DPR-Korea, Indonesia, Maldives, Timor-Leste had similar rates (17% among previously treated cases), followed by India (15% among previously treated cases), Nepal (11.7% among previously treated cases), Myanmar (10% among previously treated cases) and Sri-Lanka (1.6% among previously treated cases). Unfortunately, the MDR-TB incidence rate in Malaysia was not cited in the report. The data of the present study showed that 4.76% (56/1177) of the isolates were mono-drug resistant TB, while 2.13% (25/1177) were poly-drug resistant TB in which 19 (1.62%) were MDR-TB and 1 confirmed XDR-TB. The figures were significantly lower as compared with the study of Azura et al. [19] reporting mono-drug resistant *M. tuberculosis* isolates of 32.65%, 14.29% poly drug resistant and 8.16% of MDR-TB.

Mono-drug resistant TB to rifampicin is an emerging problem, particularly in patients with TB-HIV co-infections [20]. Although rifampicin is associated with the lowest occurrence of naturally occurring resistant mutations as compared with isoniazid [21], many of the rifampicin mono-resistant TB cases were also resistant to isoniazid and hence rifampicin resistance is frequently used as a proxy for multidrug-resistant TB (MDR-TB) [22]. In this study, 44.64% of the mono-resistant MTC were resistant to iisoniazid, 33.93% to ethambutol and only 10.72% were resistant to rifampicin and streptomycin, respectively. Mukinda et al. [23] reported that rifampicin mono-resistant-TB cases in South Africa more than tripled, from 31 cases in 2004 to 98 cases in 2008 with a calculated doubling time of 1.63 cases per year. In this study, there was no significant increase of rifampicin mono-resistant MTC.

In conclusion, *M. tuberculosis* remains the leading mycobacterial species isolated from respiratory specimens. From the laboratory perspective, NTM are important in patients with respiratory illness; they constitute about 32.55% of the mycobacterial species identified in this study. Although the majority of NTM could be identified to species levels, additional molecular assays are needed to identify and speciate NTM not interpretable by the current Haine GenoType assays. The rate of drug-resistant TB is low; continuing the monitoring of the drug-resistant isolates at the laboratory could provide a better treatment modality for the patients and help in monitoring the emergence of MDR-TB and XDR-TB in the country.

Conflict of interest

None declared.

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